

## Minireview

## RNA silencing in fungi: Mechanisms and applications

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**Abstract** Two RNA silencing-related phenomena, quelling and meiotic silencing by unpaired DNA (MSUD) have been identified in the fungus *Neurospora crassa*. Similar to the case with the siRNA and miRNA pathways in *Drosophila*, different sets of protein components including RNA-dependent RNA polymerase, argonaute and dicer, are used in the quelling and MSUD pathways. Orthologs of the RNA silencing components are found in most, but not all, fungal genomes currently available in the public databases, indicating that the majority of fungi possess the silencing machinery. Advantage and disadvantage of RNA silencing as a tool to explore gene function in fungi are discussed. © 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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## 1. The kingdom of the fungi

The kingdom of the fungi is a large and diverse group of eukaryotic organisms that includes about 100 000 known species, and perhaps a million yet to be described. As a group, the fungi have enormous impact on human affairs and the functioning of ecosystems. Fungi are the primary decomposers in the planet's ecosphere and have vital roles in nutrient recycling. Certain groups of fungi (e.g., mycorrhizal fungi) have symbiotic association with plants or algae, in which the fungus obtains carbohydrates from the photosynthetic partner, and in turn, it provides mineral ions and water. Over 90% of land plants are supposed to have a fungus associated with their roots and many would not survive without their fungal partner.

Besides being used directly as foods (e.g., mushrooms), fungi include important agents for the production of fermented foods (e.g., wine, bread, cheese and soy sauce), and for the industrial manufacture of enzymes and antibiotics. Some fungi have parasitic life cycles and cause a wide variety of diseases in animals, humans, and plants. The threat of human fungal infections is gaining public attention because of the growing number of immuno-deficient patients. Fungi are the most important group of plant pathogens, causing serious losses in crop yield and marketability worldwide.

Several species, e.g., *Neurospora crassa*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Aspergillus nidulans*, have provided sophisticated genetic systems for basic science because their cellular structures, metabolic mechanisms and gene organization are similar to those of higher eukaryotes such as plants and animals. Their advantages as model organisms stem from the exceptional ease with which fungi can be genetically and molecularly manipulated, combined with their smaller haploid genomes and simple and short life cycles. Research on those fungi is at the cutting edge of many scientific fields including population and molecular genetics, cell biology, development, DNA repair, photobiology, circadian rhythms, evolution, and gene silencing.

## 2. Quelling, a gene silencing phenomenon discovered in the fungus *N. crassa*

Quelling is a gene silencing phenomenon first described in *N. crassa* in 1992 [1]. The genetic and biochemical features of quelling indicate that quelling belongs to the broad category of RNA-mediated gene silencing mechanisms that includes post-transcriptional gene silencing (PTGS) or co-suppression in plants and RNA interference (RNAi) in animals; here, I collectively refer to these mechanisms as “RNA silencing”. Quelling was originally described as reversible inactivation of gene expression by transformation with repeated homologous sequences. Quelling occurs during the vegetative phase of growth and, like for co-suppression in plants, it affects both transgenes and endogenous genes. While DNA methylation is often correlated with quelling, it is not required for the process [2]. Quelling is dominant in heterokaryons with nuclei from “quelled” and wild-type strains, an intriguing feature of that suggested that a mobile signal, e.g., RNA, acts in trans to cause silencing [2]. Indeed, a series of studies on quelling-deficient (*qde*) mutants of *N. crassa* has brought remarkable progress and implicated small interfering RNAs in the quelling mechanism. Showing that the *qde-1* mutant was defective in an RNA-dependent RNA polymerase (RdRP) [3], suggested the existence of RNA components for the quelling pathway and related eukaryotic RNA silencing pathways. This was supported by the findings that the *SDE1/SGS2* gene in *Arabidopsis* and the *ego-1* gene in *Caenorhabditis elegans*, both of which encode RdRP, are required for PTGS and RNAi, respectively [4]. Thus, the genetic evidence suggested that RNA silencing phenomena share an evolutionarily conserved machinery. Similarly, the protein product encoded by the second *qde* gene, *qde-2* was shown to be a piwi-PAZ domain (PPD or

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Argonaute) protein [5], which is an essential and conserved component of the RNA silencing pathway in a variety of eukaryotic organisms. The third *qde-3* encodes a putative RecQ-type DNA helicase [6]. Recently, the *Neurospora* QDE-3 RecQ helicase and its homologue, RecQ-2, have been shown to play a role in recombination repair [7,8], suggesting that QDE-3 RecQ helicases may have a dual role in *N. crassa*. These work on quelling in *Neurospora* set the stage for our understanding of the basic components of the RNA silencing machinery.

### 3. Meiotic silencing by unpaired DNA, a novel RNA silencing-related phenomenon

A new RNA silencing-related phenomenon, called meiotic silencing by unpaired DNA (MSUD), which was originated from the study on meiotic transvection of the *Asm-1* gene [9], was uncovered in *N. crassa* [10]. While *N. crassa* is haploid during vegetative growth, it has a transient diploid cell, when two haploid nuclei of opposite mating type fuse to form the zygote. The zygote undergoes meiosis, which involves pairing of homologous chromosomes, followed by post-meiotic mitosis that results in the production of asci with eight haploid ascospores. MSUD abolishes the expression of genes that exist in one parental chromosome but not in its pairing partner and, therefore, cause unpaired DNA during meiosis. Interestingly, MSUD affects not only the unpaired copies but rather any copy of the unpaired gene in the genome even if the additional copies are paired [9]. This suggested that a mobile trans-acting signal is involved in MSUD as is the case in quelling.

MSUD can be visualized by making use of gene fusions with green fluorescent protein (GFP), e.g., histone H1-GFP (hH1-GFP). When both parent strains possess the same hH1-GFP locus, hH1-GFP is expressed at any stage during meiosis and ascospore development (Fig. 1A) [11]. However, when wild-type (no hH1-GFP locus) and the hH1-GFP strain are crossed, hH1-GFP is silenced by MSUD during meiosis (Fig. 1B; [11] and Raju, Shiu, Freitag and Metzner, unpublished results). The silenced hH1-GFP, however, begins to be reexpressed approximately 12–24 h after spore delimitation. Thus, MSUD in heterozygous asci operates for a limited period of time from

an early stage of meiosis after karyogamy to ascospore maturation.

Unexpected connections between DNA pairing and RNA silencing were uncovered by genetic screens for suppressors of meiotic transvection and MSUD [10]. One semidominant *Neurospora* mutant, *Sad-1* (suppressor of ascus dominance-1) is shown to be deficient in MSUD. This mutation also suppresses the sexual phenotypes of many ascus-dominant mutants that might be caused by failure of meiotic pairing, and even complements, albeit partially, the sterility of interspecific crosses that may involve unpaired DNA due to chromosomal variation. Using the UV-induced *sad-1* mutant, the *Sad-1* gene was isolated and revealed to encode RdRP similar to QDE-1 [10], suggesting that MSUD involves a molecular mechanism similar to RNA silencing. This is consistent with the fact that MSUD is reversible and involves a mobile trans-acting signal for silencing. The isolation of two additional suppressor loci of MSUD, *Sms-2* (suppressor of meiotic silencing-2) and *Sms-3* (suppressor of meiotic silencing-3), lend support to this because *Sms-2* and *Sms-3* encode paralogs of QDE-2 (PPD protein) and DCL-2 (Dicer), respectively [12,13]. Intriguingly, different sets of protein components are required for MSUD and quelling, which indicate that two separate silencing pathways exist in *N. crassa* [13] (Fig. 2). In *N. crassa*, the two dicer-like proteins, DCL-2 and SMS-3 (DCL-1) were reported to be redundantly involved in the quelling pathway [14]. However, DCL-2 appeared to have stronger activity to produce siRNAs in vitro [14]. In *Magnaporthe oryzae* (formerly *M. grisea*) [15], a fungus closely related to *N. crassa*, it has been shown that one dicer-like protein, MDL-2 (DCL-2 ortholog) is solely responsible for siRNA biogenesis [16]. Therefore, it appears possible that DCL-2 is the primary dicer protein responsible for the quelling pathway in *N. crassa* even though DCL-1 can compensate when DCL-2 is lost.

### 4. RNA silencing pathways in fungi and higher eukaryotes

In higher eukaryotes, two related but distinct RNA silencing pathways are known. Based on the small RNA molecules involved, those are referred to as “small interfering RNA (siRNA)-directed pathway” and “microRNA (miRNA)-directed

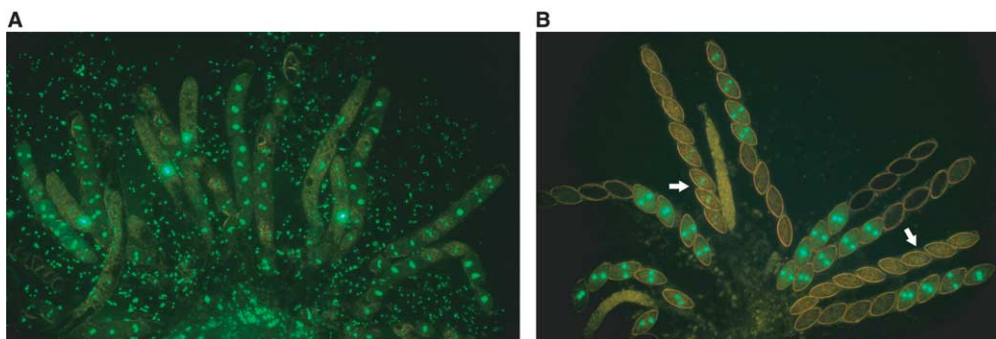


Fig. 1. Fluorescence images of *N. crassa* asci showing expression of GFP-tagged histone H1 (hH1-GFP). (A) A rosette of asci from hH1-GFP × hH1-GFP at various stages of development. When homozygous, hH1-GFP is expressed throughout meiosis and ascospore development (no meiotic silencing). (Reproduced from [11], with permission of the publisher and authors.) (B) A small rosette of maturing asci from Wild-type × hH1-GFP. In the heterozygous asci, histone H1-GFP was silenced during meiosis and until after ascospore delimitation because of MSUD (indicated by arrows). The silenced hH1-GFP, however, begins to be reexpressed approximately 12–24 h after spore delimitation. The GFP-tagged nuclei in four of the eight ascospores (two nuclei per spore at this stage) begin to glow about 20–24 h after spore delimitation. The remaining ascospores contain untagged nuclei from the wild-type parent. (Photo credit: Namboori B. Raju, Stanford University.)

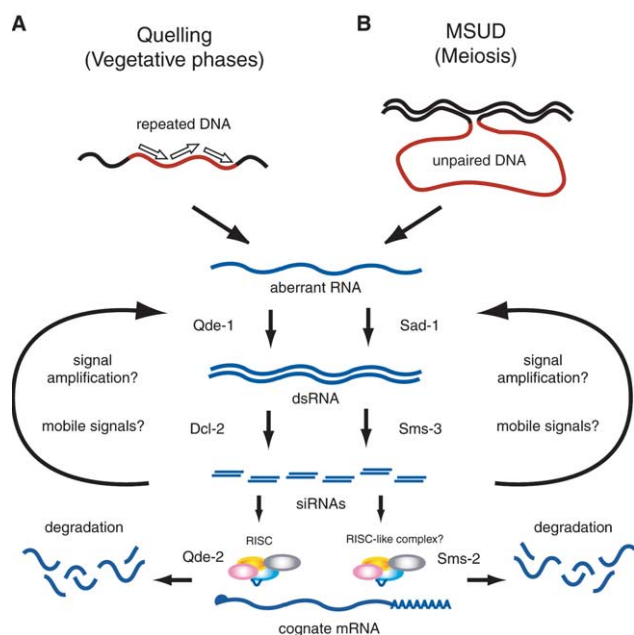


Fig. 2. A proposed model of two RNA silencing pathways in *N. crassa*. (A) During the vegetative phases of the *N. crassa* life cycle, repeated sequences in the genome can induce quelling. In this pathway, dsRNA produced by Qde-1 RNA-dependent RNA polymerase is diced into siRNAs mainly by the action of Dcl-2. The siRNAs guide degradation of cognate mRNA after their incorporation into RNA-induced silencing complex (RISC) where Qde-2 is one of the components. (B) During meiosis, a DNA fragment that has failed in pairing (unpaired DNA) triggers the second RNA silencing pathway in *N. crassa*, called MSUD. Mechanisms of silencing in MSUD are supposed to be quite similar to those in quelling except that MSUD uses a different set of silencing protein components (paralogs) from those in the quelling pathway.

pathway". Both siRNAs and miRNAs interfere with gene expression through targeted degradation of mRNA or through the suppression of translation. The siRNAs act as guides for an siRNA-induced silencing complex (siRISC) to target perfectly complementary mRNAs for degradation. The second class of small RNAs, miRNAs is processed from imperfect stem-loop RNA precursors (pre-miRNAs) that are transcribed from non-protein-coding genes within plant and animal genomes. The mature miRNA causes the translational repression of the target mRNA transcript that has imperfect complementarity to the miRNA. It should be noted that miRNA can also direct cognate mRNAs for degradation when the sequence complementarity is perfect [17]. Most of the known miRNAs so far are involved in growth and development.

Recently, it has been shown in *Drosophila* that different sets of proteins are responsible for the siRNA and miRNA pathways, respectively [18,19]. In the *Drosophila* genome, paralogs of dicer protein, *Dcr-1* and *Dcr-2*, and those of PPD protein, *AGO-1* and *AGO-2* are identified. By characterizing *Drosophila* *Dcr-1* and *Dcr-2* mutants, Lee et al. [18] demonstrated that mutation in *Dcr-1* blocks processing of miRNA precursors whereas *Dcr-2* mutants are defective for processing siRNA precursors [18]. Similarly, Okamura et al. [19] showed that *Drosophila* embryos lacking *AGO-2* are defective in siRNA-directed RNAi but are still capable of miRNA-directed target RNA cleavage [19]. *AGO-1* is, in contrast, dispensable for siRNA-directed target RNA cleavage but is essential for mature

miRNA production. Therefore, it seems that in *Drosophila* the miRNA pathway is mediated by one set of silencing protein components (including *Dcr-1* and *AGO-1*), while an other set of proteins (including *Dcr-2* and *AGO-2*) is responsible for the siRNA pathway. Also in plants, involvement of distinct dicer proteins in siRNA and miRNA biogenesis has been demonstrated [20].

In *Neurospora*, a distinct set of silencing protein components seems to be responsible for each of the quelling and MSUD pathways [13] (Fig. 2). Quelling is the siRNA-directed silencing pathway in *Neurospora* that is evolutionarily conserved but no miRNA-directed silencing pathway has been so far identified in fungi. Does the MSUD pathway in *Neurospora* correspond to the miRNA pathway in higher eukaryotes? MSUD and miRNA-directed silencing may have independently arisen in fungi and higher eukaryotes, respectively, or the MSUD and miRNA pathways may have evolved from an ancient silencing machinery as both are implicated in development. In this regard, intriguing reports have recently emerged describing that MSUD-like phenomena occur in *C. elegans* and mouse [21,22]. Using the RNA-FISH method coupled with immunohistochemical analysis, Turner et al. [22] showed that unsynapsed regions of a translocated autosome in the X chromosome were transcriptionally silenced during meiosis in mouse cells whereas synapsed euchromatin is actively transcribed [22]. Similarly in *C. elegans*, it has been shown that DNA lacking a pairing partner during meiosis is targeted for methylation of histone H3 at Lys9 (H3-Lys9) and transcriptionally silenced [21]. Since the silencing of unsynapsed meiotic chromosomes in mouse and *C. elegans* is not post-transcriptional but rather transcriptional and concomitant with chromatin modification, it appears that a mechanism different from MSUD is involved in those silencing phenomena. Nevertheless, some as of yet unknown surveillance mechanism for unpaired DNA seems to be conserved in a wide range of eukaryotic organisms. It is tempting to speculate that the RNA silencing machinery might generally play some role in the genomic surveillance for unpaired DNA since the machinery is also known to be involved in transcriptional silencing by siRNA-mediated histone methylation in a diverse of organisms such as fission yeast, *Drosophila* and *Arabidopsis* [23]. It should be noted, however, that there is no indication that DNA methylation and chromatin modification are involved in MSUD or quelling in *Neurospora* [24].

## 5. RNA silencing in fungi other than *Neurospora*

Until recently, only a limited number of post-transcriptional gene silencing phenomena had been reported in fungi other than *Neurospora*. These included a co-suppression-like phenomenon in *Cladosporium fulvum* [25] and internuclear gene silencing in *Phytophthora infestans* [26], which belongs to the *Oomycota* and therefore not a true fungus. However, after the discovery of RNAi in 1998 [27], attempts were made to harness this technology for controlling gene expression in a variety of fungal species. Consequently, suppression of gene expression by a dsRNA-expressing plasmid or related-system has been shown in many fungal species including *Ascomycota*, *Basidiomycota*, and *Zygomycota* [28–34], and as well as the fungus-like *Oomycota* [35]. Involvement of typical RNA silencing protein components such as dicer in the silencing phenomena was shown in *A. nidulans*, *M. oryzae* (*M. grisea*), *N. crassa*,



and *S. pombe* [3,14,16,29,36], and biogenesis of siRNA was detected in *A. nidulans*, *M. oryzae*, *Mucor circinelloides*, *N. crassa*, and *S. pombe* [29,30,33,36,37]. Therefore, the fundamentals for RNA silencing seem to be conserved in most of fungal species with some exceptions described below.

Recent advances in fungal genomics enable a comparative genomics approach. Searches for the typical RNA silencing components in the public fungal genome databases resulted in the identification of RdRP-, PPD- and dicer-like proteins in various fungi belonging to *Ascomycota*, *Basidiomycota*, and *Zygomycota* but failed in a subset of fungi including the ascomycetes *S. cerevisiae* (budding yeast), *Candida guilliermondii*, and *C. lusitaniae*, and the basidiomycete *Ustilago maydis* (Nakayashiki et al. submitted, Fig. 3). *Candida albicans*, and *C. tropicalis* also apparently lack dicer- and RdRP proteins in their genomes. Thus, the RNA silencing machinery may have been lost in a portion of fungal species during evolution. This assumption was supported by the facts that relics of the RNA silencing genes were identified in several fungal genomes such as *A. nidulans* and that the loss of the RNA silencing machinery was observed in taxonomically distant fungal species.

To gain an insight into the molecular evolution and diversity of the RNA silencing machinery among fungi, phylogenetic analysis of RdRP-, PPD- and dicer-like proteins was performed with representative fungal species comprising five ascomycetes *A. nidulans*, *Fusarium graminearum*, *M. oryzae*, *N. crassa*, and *S. pombe*, three basidiomycetes *Cryptococcus neoformans* (JEC21 strain), *Phanerochaete chrysosporium* and *Coprinus cinereus*, and a zygomycete *Rhizopus oryzae*. *Arabidopsis thaliana* (plant), and *Drosophila melanogaster* (fruit fly) were used as outgroup members when available (Fig. 3). In the model fungus, *N. crassa*, three paralogs of RdRP-, two of PPD-, and two of the dicer-like proteins have been identified. The ascomycete fungi used here appear to mostly follow the standard gene composition in *Neurospora* even though a loss or expansion of the genes is often observed. *S. pombe* has only one set of RdRP-, PPD- and dicer-like proteins. The other paralogs in *S. pombe* might be lost. Alternatively, *S. pombe* might retain a master set of the silencing component, and their paralogs in the other ascomycete fungi may occur by duplication and following diversification of the master set. It is noteworthy that all silencing component proteins present in *S. pombe* are more closely related to the *N. crassa* counterparts involved in MSUD compared to the ones for quelling. *A. nidulans* also seems to possess only one dicer- and PPD-like protein each due to loss of their paralogs since the gene relics can be observed in the genome [29]. Conversely, expansion of RdRP- and PPD-like genes appears to have occurred in some of the ascomycete fungi. In the *F. graminearum* genome, one ortholog each of *N. crassa* sad1 and rrp3 is recognizable whereas there are two RdRP-like paralogs (FG1 and FG4) closely related to *N. crassa* qde1, suggesting that the qde1 orthologs may be arisen by gene expansion. Similarly, *M. oryzae* has two PPD-like proteins (MG1 and MG3) in the phylogenetic branch to which *N. crassa* qde-2 belongs. Nevertheless, with the exception of the FG5 RdRP gene in *F. graminearum* (Fig. 3B), all the ascomycete genes involved in the RNA silencing seem to have a *N. crassa* ortholog, therefore, indicating that they mostly share similar molecular fundamentals.

In the basidiomycetes, at least two distinct classes of dicer-, three of RdRp-, and two of PPD-like proteins are recognizable (Fig. 3). The numbers of the protein classes appear to corre-

spond to those in the ascomycetes. In fact, two groups of paralogous proteins from the basidiomycetes and ascomycetes form a single independent cluster in some cases (e.g., the Dcl-2, Rrp3, and Qde-2 clusters), indicating that those proteins may have arisen before the diversification of *Basidiomycota* and *Ascomycota*. In the other cases, however, relationships between the groups of the proteins from the basidiomycetes and ascomycetes cannot be resolved due to the low bootstrap values for the deeper branches of the trees.

Compared to the case with the ascomycetes, more extensive gene expansion and a wider diversity in the silencing-related proteins are observed in the basidiomycetes used here. Especially, the proteins in *C. neoformans* seemed to be genetically distant from their counterparts in the other basidiomycetes, indicating that the RNA silencing machinery might be distinctively evolved in *C. neoformans*. Interestingly, *C. neoformans* carries unique dicer proteins that lack the DEAD/DEAH box helicase, a typical signature of dicer proteins. Helicase-lacking dicer protein (dcl1p) has also been found in *Tetrahymena*, and shown to play a crucial role in the RNA silencing-related phenomenon, internal eliminated sequences (IES), by processing dsRNA into siRNA-like small RNAs, termed scan RNAs (scnRNAs) [38]. Therefore, *C. neoformans* dicer proteins are unusual but may still serve the same function.

Extensive gene expansion seems to occur with PPD-like protein in *P. chrysosporium* (Fig. 3C). Similar gene expansion is also observed with basidiomycete RdRP proteins (CC1 and CC2, PC1 and PC2) (Fig. 3B and C). It should be noted that some RdRP- and PPD-like proteins especially in *C. cinereus* were eliminated from the phylogenetic analysis because of weak homology to the conserved motives. Therefore, it could be possible that more dramatic gene expansion and diversification might take place in the basidiomycetes. One should take also into account that these results could be biased due to the limited sequence information currently available in public databases. In the zygomycete *R. oryzae*, expansion of RdRP genes also appears to have occurred since closely related RdRP-like proteins are identified in two distinct branches (Fig. 3B).

## 6. RNA silencing as a tool for exploring gene function in filamentous fungi

Because of the compact and small-sized genome, there are a relatively large number of fungal species whose genomes have been completely sequenced. Those include the saprobic model organisms *A. nidulans*, *N. crassa*, *S. pombe*, the industrial fungi *Aspergillus oryzae*, *S. cerevisiae*, and the plant pathogens *F. graminearum*, *M. oryzae* (*M. grisea*), *U. maydis* as well as the animal pathogens, *Aspergillus fumigatus*, *C. albicans*, and *C. neoformans*. More fungal species or multiple genomes from within single fungal species are currently being sequenced. To take full advantage of this wealth of genetic information for unraveling how the genes work, RNA silencing is one of the most powerful approaches. This approach has been rapidly developed and employed in higher eukaryotes even on a genome-wide scale as shown in *D. melanogaster*, and *C. elegans*, and is currently under way in *Arabidopsis* (AGRIKOLA project; <http://www.agrikola.org/>) and even human [39]. Since the operation of RNA silencing has been shown to exist in

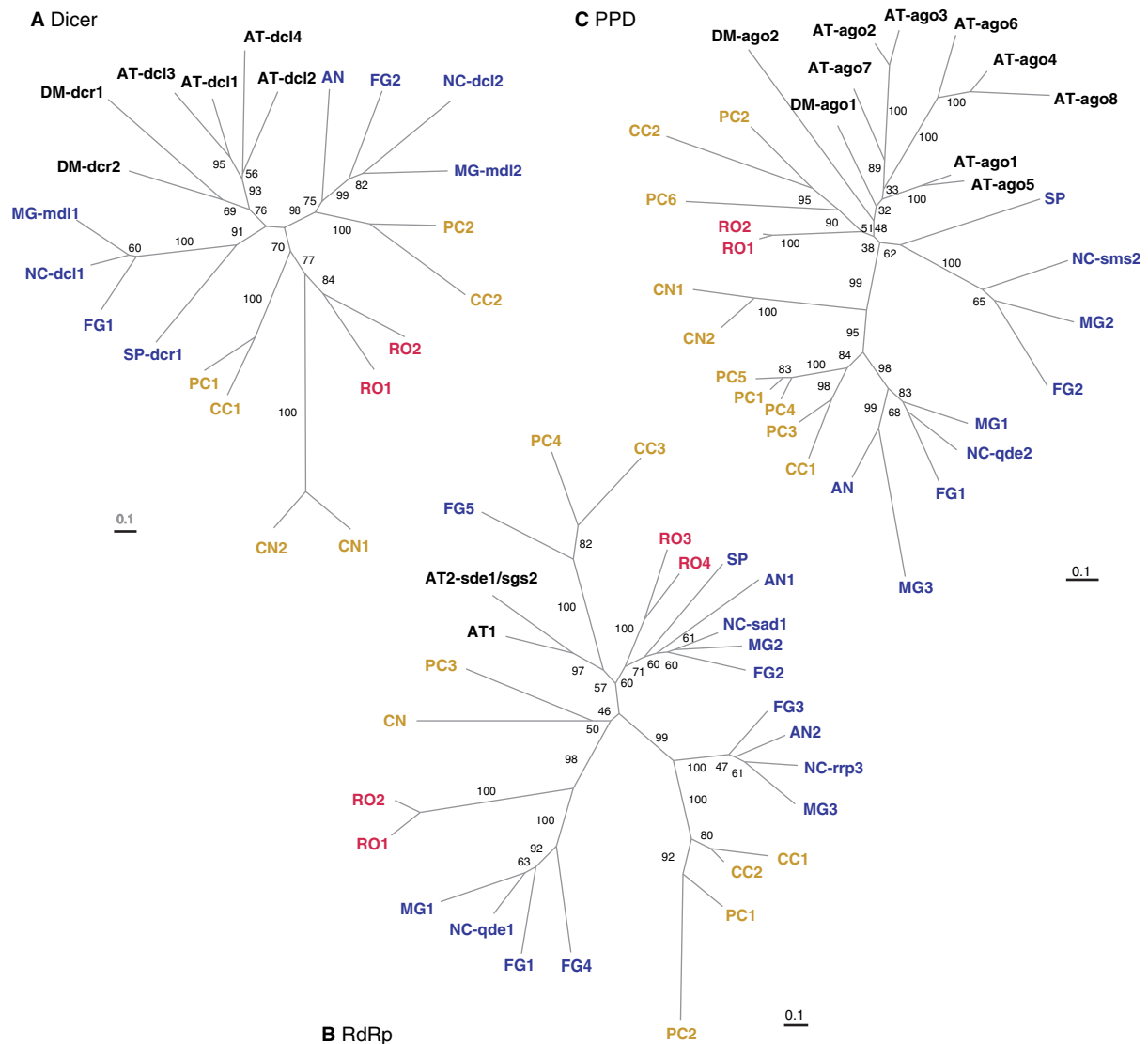


Fig. 3. Radial phylogenetic trees of dicer- (A), RNA-dependent RNA polymerase (RdRp)- (B), and piwi-PAZ (PPD)-like proteins (C). Amino acid alignments were generated by Clustal W using sequences from the RNaseIII (A), RdRP (B), and piwi (C) domains in the proteins. All distance trees were constructed by the Neighbour-Joining method and their robustness was estimated by performing 1000 bootstrap replicates (expressed as percentages). Proteins from the ascomycetes, basidiomycetes and zygomycete are indicated in blue, brown and red, respectively. (A) AN, *Aspergillus nidulans* (AN3189); CC1, *Coprinus cinereus* (contig 1.114); CC2, (contig 1.321); CN1, *Cryptococcus neoformans* (CNC03670); CN2, (CNC03680); FG1, *Fusarium graminearum* (FG09025); FG2, (FG04408); MG-mdl1, *Magnaporthe oryzae* (grisea) (MG01541); MG-mdl2, (MG07167); NC-dcl1 (Sms-3), *Neurospora crassa* (NCU08270); NC-dcl2, (NCU06766); PC1, *Phanerochaete chrysosporium* (whiterot1:70591); PC2, (whiterot1:94166); RO1, *Rhizopus oryzae* (contig 1.1); RO2, (contig 1.9); SP-dcr1, *Schizosaccharomyces pombe* (SPCC188.13c); AT-dcl1, *Arabidopsis thaliana* (At1g01040); AT-dcl2, (At3g0330); AT-dcl3, (At3g43920); AT-dcl4, (AT5G20320); DM-dcr1, *Drosophila melanogaster* (AAF56056); DM-dcr2, (NP\_523778). (B) AN1, (AN4790); AN2, (AN2717); CC1, (contig 1.101); CC2, (contig 1.25); CC3, (contig 1.250); CN, (CNG01230); FG1, (FG06504); FG2, (FG08716); FG3, (FG01582); FG4, (FG04619); FG5, (FG09076); MG1, (MG07682); MG2, (MG02748); MG3, (MG06205); NC-qde1, (NCU07534); NC-sad1, (NCU0217); NC-rrp3, (NCU08435); RO1, (contig 1.38); PC1, (scaffold 19); PC2, (scaffold 41); PC3, (scaffold 129); PC4, (scaffold 135); RO2, (contig 1.4); RO3, (contig 1.28); RO4, (contig 1.37); SP, (SPAC6F12); AT1, (At4g11130); AT2-sde1/sgs2, (At3g49500). (C) AN, (AN1519); CC1, (contig 1.198); CC2, (contig 1.325); CN1, (CNJ00490); CN2, (CNJ00610); FG1, (FG08752); FG2, (FG00348); MG1, (MG01294); MG2, (MG11029); MG3, (MG10003); NC-qde2, (NCU04730); NC-sms2, (NCU09434); PC1, (whiterot1:25985); PC2, (whiterot1:30243); PC3, (whiterot1:68037); PC4, (whiterot1:70167); PC5, (whiterot1:81651); PC6, (whiterot1:96257); RO1, (contig 1.1); RO2, (contig 1.30); SP, (SPCC736.1); AT-ago1, (At1g48410); AT-ago2, (At1g31280); AT-ago3, (At1g31290); AT-ago4, (At2g27040); AT-ago5, (At2g27880); AT-ago6, (At2g32940); AT-ago7, (At1g69440); AT-ago8, (At5g21030); DM-ago1, (NP\_725341); DM-ago2, (Q9VUQ5).

many fungi, RNA silencing is opening new avenues to explore the genomes of the fungi.

To date, RNA silencing in fungi has mostly been induced by plasmid constructs that express hairpin RNA, sometimes with an intron sequence at the loop structure. To efficiently

construct vectors of this type by PCR-based cloning, we have developed the versatile vector pSilent-1 for ascomycete fungi, which carries a hygromycin resistance cassette and a transcriptional unit for hairpin RNA expression with multiple cloning sites and a spacer of an intron sequence [40]. We

showed that *M. oryzae* endogenous genes such as *mpg1* and a polyketide synthase-like gene were silenced at varying degrees by pSilent-1-based vectors in 70–90% of the resulting transformants. Ten to fifteen percent of the silenced transformants exhibited almost “null phenotype”. This vector was also efficiently applicable to silence a GFP reporter in another ascomycete fungus *Colletotrichum lagenarium* [40]. Therefore, pSilent-1 may serve as an efficient reverse genetic tool in a wide range of ascomycete fungi, at least for gene analysis on small or moderate scale.

## 7. Advantages and disadvantages of RNA silencing over the knock-out strategy

Compared with conventional gene knock-out strategies, the RNA silencing approach has potential advantages. One major advantage of RNA silencing is its applicability to downregulate gene expression without regard for gene targeting efficiency. The efficiency of homologous recombination varies considerably among fungal species. Gene targeting efficiency is relatively high in the model fungi such as *A. nidulans*, *N. crassa*, and *M. oryzae*, but this is not the case in all fungi. The majority of fungi consist of multicellular or multinuclear hyphae, and some of them have two or more genetically different nuclei in a common cytoplasm (heterokaryon). These characteristics of fungi make gene targeting complicated and inefficient. Since RNA silencing is locus-independent and mediated by a mobile trans-acting signal in the cytoplasm, it can be applicable to fungi with low gene targeting efficiency or even to fungal species such as zygomycetes, which has the tubular hyphae containing many nuclei inside one cell partition. Secondly, RNA silencing allows flexibility in gene inactivation experiments since it induces gene suppression in a sequence-specific, but not locus-specific, manner. For example, simultaneous silencing of homologous genes or even heterologous genes has been demonstrated by targeting a conserved sequence of a gene family or by constructing a chimeric sequence derived from different genes [28,31,41,42]. RNA silencing with an inducible promoter or transient silencing by siRNA allows study of gene expression at a specific stage during development or how it affects different parts of the organism. This kind of study cannot be achieved by gene deletion strategies because they eliminate the targeted gene permanently. In addition, it has been shown that RNA silencing can be used to selectively degrade specific alternatively spliced mRNA isoforms in cultured *Drosophila* cells [43]. Therefore, RNA silencing offers various options for specific knock-down of a gene with less effort. Finally, RNA silencing can be used for analyses of lethal genes since it mostly induces “knock-down” of gene expression but not complete “knock-out”. The function of essential genes in various aspects of biological processes remains largely unknown since classical genetic approaches such as mutant screening are not available because of lethality. Imperfect silencing with reduced levels of gene expression would shed light on unexpected roles of essential genes in fundamental biological phenomena.

The major disadvantage of RNA silencing is that an incomplete and/or reversible mutation sometimes makes experimental results difficult to interpret. Often times it is reported that there is a phenotypic difference between knock-out and knock-down mutants [44]. Actually, the *mpg1*

knock-out mutant of *M. oryzae* is known to lose the pathogenicity completely. However, most of *mpg1* knock-down mutants by a pSilent-1-based vector retained pathogenicity, albeit reduced, at varying degrees (unpublished data). Therefore, particular attention should be paid to phenotypic discrepancies when we interpret data obtained by RNA silencing experiments. Secondly, a fraction of genes and certain cell types such as neurons and sperm are known to be resistant to RNA silencing for as yet unknown reasons. Although, in fungi, no cell type or gene has been reported to be resistant to RNA silencing so far, this may be the case with certain fungal cells or genes. Thirdly, although RNA silencing operates in a sequence-specific manner, several studies have suggested that the specificity of silencing is not absolute. This causes unexpected changes in gene expression patterns, called off-target effects. Recent microarray studies suggested that siRNAs can provoke off-target effects by as few as 14 base pairings between the siRNA and its target [45]. Lastly, RNA silencing in fungi research is currently lacking in genetic tools and information. Especially, no high-throughput system for the RNA silencing approach is established in fungi. In higher eukaryotes, various such systems are available, for example, RNAi by feeding, virus-induced gene silencing (VIGS), RNAi by synthetic siRNA, and high-throughput silencing vectors (e.g., pHELLSGATE, and the silencing by the heterologous 3'-untranslated region (SHUTR) system for plants [46,47]; various commercial vectors with convergent opposing promoters for animals). Establishment of one of these methods is essential for the use of genome-wide RNA silencing in fungus research.

## 8. Concluding remarks

In fungi, the conventional gene knock-out strategy by homologous recombination is currently more widely used to identify gene function than an RNA silencing approach. This strategy has been successfully employed in genome-scale analysis of gene function in budding yeast *S. cerevisiae* [48]. Genome-wide gene disruption experiments are underway in *N. crassa* with mus-51 and mus-52 mutants that allow homologous recombination with almost 100% efficiency [49]. As described above, RNA silencing and gene knock-out approaches have different advantages; thus, a combined approach of RNA silencing and gene knock-out technologies will without doubt greatly facilitate exploring gene function in fungi in the post-genomics era.

As demonstrated by the discovery of quelling and MSUD in *Neurospora*, the unique feature of the fungi may provide an excellent model for detailed studies of the molecular machinery of RNA silencing. Recent demonstration of the RITS component in *S. pombe* [50] has also contributed to our understanding of a connection between the RNA silencing machinery in the cytoplasm and chromatin modification in the nucleus. Despite the rapid progress in understanding the molecular mechanisms of RNA silencing, intriguing questions such as how different RNA silencing pathways are originated, what is the signal of aberrant RNA to be recognized by RdRP, and how different sets of the paralogue proteins are directed into the distinct RNA silencing pathways, still remain to be answered. The small organisms could bring further big surprises.

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